EXHIBIT 10

Viruses and Diabetes

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ABSTRACT: **Insulin-dependent diabetes mellitus (IDDM) is a multifactorial disease. Besides a genetic predisposition environmental factors have been implicated in the pathogenesis of β cell destruction. Among these environmental factors viruses have been the focus of many studies. Some viruses are diabetogenic in animals, and others have been implicated as triggers in human IDDM by temporal and geographical association between IDDM and viral infections, serological evidence of infection in recently diagnosed diabetic patients, and the isolation of viruses from the pancreas of affected individuals. We discuss possible pathomechanisms of viral infections in β cell destruction and review the studies on involvement of enteroviruses, retroviruses, rubella viruses, cytomegaloviruses, and Epstein-Barr viruses in human IDDM. We also report on studies of diabetogenic viruses in animal models as well as on viral infections protecting from IDDM. Some of the difficulties in linking viral infections to IDDM will be illustrated with data from a transgenic mouse model in which IDDM can be precipitated by infections with certain strains of lymphocytic choriomeningitis virus (LCMV). Emerging treatment concepts that do not rely on defining the initiating autoantigens but involve self-reactive regulatory lymphocytes such as oral antigen administration, as well as DNA vaccines, will be discussed briefly.**

KEYWORDS: **IDDM; viral infection; enterovirus; Coxsackie virus; retrovirus; rubella virus; cytomegalovirus; Epstein-Barr virus; LCMV; regulatory T-cells; oral tolerance; DNA vaccines**

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is a multifactorial disease associated with a strong genetic predisposition.[1,2] Nevertheless, only a relative small proportion—less than 10%—of genetically susceptible individuals progress to clinical disease. This implies that additional factors are needed to trigger beta cell destruc-

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TABLE 1. Viruses implicated in the pathogenesis of IDDM in humans and other species

Virus	Host	Genetic factors	Remarks
RNA viruses			
Picornaviridae			
Coxsackie B	mice	+	virus passaged in murine β cells before infection
	nonhuman primates	+	virus passaged in monkey β cells before infection
	humans	ND	evidence from epidemiological studies; anecdotal reports; virus identified and isolated from pancreas of IDDM patients shown to be diabetogenic in mice
Encephalomyocarditis	mice	+	cytolytic destruction of β cells leading to clinical diabetes
	hamster	+	
Mengovirus	mice	+	cytolytic destruction of β cells
Foot-and-mouth disease virus	pigs, cattle	ND	
Retroviridae			
Endogenous retroviruses	mice	+	β cell–specific expression of retroviral genes associated with development of insulitis and IDDM in NOD mice
	humans	+	retrovirus-like particles identified in β cells of IDDM patients cross-reactive antibodies with retroviral antigens identified in IDDM patients and close relatives retroviral genome with superantigen in envelope area cloned from IDDM patients, but no viral particles isolated so far and no correlation with disease found in several studies
Togaviridae			
Rubella	hamsters	ND	possible association with autoimmune IDDM
	humans	ND	possible association with autoimmune IDDM, especially congenital rubella syndrome
Flavivirdae			
Bovine viral diarrhea-mucosal disease virus	cattle	ND	suspected autoimmune response to β cells
Paramyxoviridae			
Mumps virus	humans	+	possible induction of islet cell autoantibodies; case reports
Reoviridae			
Reovirus	mice	+	possible association with autoimmunity and diabetes in mice

TABLE 1. Viruses implicated in the pathogenesis of IDDM in humans and other species (*continued*)

Virus	Host	Genetic factors	Remarks
DNA viruses			
Parvoviridae			
Kilham's rat virus	rats	+	no distinct infection of β cells development of β cell–specific autoimmunity leading to IDDM
Herpesviridae			
Cytomegalovirus	humans	ND	associated with autoimmune IDDM, can infect β cells
Epstein-Barr	humans	ND	possible induction of autoimmune IDDM
Varicella zoster	humans	ND	indirect evidence of association with IDDM

ND = not determined.

tion in genetically predisposed subjects. Attempts to establish a direct epidemiological association between viral infections and various autoimmune disorders have been hampered by the fact that each individual has frequently incurred multiple different viral infections by the time the disease is diagnosed. Further, immunologically mediated damage might occur after the causative viral infection has been cleared, and no viral "footprints" will be detectable in the affected organ(s). Clinical diabetes is the end stage of destructive insulitis after a sometimes prolonged period of autoimmune destruction. It has been estimated that at the time of diagnosis only 10–20% of the insulin-producing beta cells are still functioning. Environmental factors have been suggested to be involved in the pathogenesis of type I diabetes both as triggers and potentiators of beta cell destruction.[3]

Several lines of evidence support an important role for environmental factors in the pathogenesis of IDDM: Concordance rates of IDDM in monozygotic twins have been reported to be just between 25 and 60%.[4–6] Furthermore, epidemiological data suggests a role of environmental factors in the pathogenesis of IDDM: There are significant variations in IDDM incidence in confined geographical areas even in populations with similar genetic backgrounds.[7] Migrant studies support a role for different environmental agents in different geographical areas, although they also underline the importance of a IDDM-susceptible genetic background, as offspring of people coming from high-risk areas still contain a markedly higher risk after the parents have moved to a low-risk environment.[8]

More evidence for an environmental influence on IDDM came from reports about an overall increase in incidence over the past decades in European populations[6,10] and secular trends in IDDM incidence over periods of some years in different European[7] and an American[11] population. Besides this, epidemic outbreaks of IDDM have been reported.[12]

It is important to note that beside viruses other environmental factors like dietary components, bacterial infections, and drugs/toxins have also been considered as possible triggers of IDDM.

TABLE 2. Possible mechanisms of β cell destruction by viruses

Direct effects of viral infection on β cells
Cytolytic effect of viral infection[a]→β cell necrosis/apoptosis
Indirect/immune mediated effects
Viral antigens expressed in β cells, persistent infection of β cells[a]
Bystander death due to infection of surrounding tissue and release of substances toxic to β cells
Altered MHC expression/expression of costimulatory molecules
Bystander activation of autoreactive lymphocytes by cytokines
Bystander activation of autoreactive lymphocytes by release of β cell antigens/altered β cell antigens
Bystander activation of antigen presenting cells (APC) by cytokines or viral infection→ activation of autoreactive lymphocytes
Molecular mimicry of viral antigens with β cell antigens
Activation and expansion of autoreactive T cells by virally encoded superantigens
Altered immune regulation caused by viral infection (regulatory T cells, natural killer cells, natural killer T cells)
Altered repertoire of memory T cells due to various viral infections and other environmental influences

[a]Tropism of viral infection to β cells.

Many viruses are diabetogenic in animals, and some have been implicated in humans by temporal and geographical association between IDDM and viral infections, serological evidence of infection in recently diagnosed diabetic patients, and the isolation of viruses from the pancreas in a few such cases (TABLE 1).

The focus of this article is to introduce novel findings that demonstrate that methods currently in use to identify viruses as a trigger for autoimmunity are likely to fall short and point out potential future approaches and strategies to identify or rule out an infectious cause. Emerging treatment concepts that do not rely on defining the initiating autoantigens but involve self-reactive regulatory lymphocytes such as oral antigen administration, as well as DNA vaccines,[13,14] will be discussed briefly.

MECHANISM OF β CELL DAMAGE BY VIRUSES

Several pathomechanisms of β cell damage by viruses have been proposed for different viruses in animal models of IDDM and in humans (TABLE 2). Although the exact mechanism is not clear for most viruses (TABLE 1), it is likely that multiple pathways are involved in the pathogenicity of a given virus.

Viruses that show an infectious tropism for β cells are able to kill the β cells during their lytic life cycle. Alternatively, the virally infected β cells become the target of the immune system by continuous expression of viral proteins or alterations of

self-antigens from β cells. Direct viral infection of the β cells is not necessarily a prerequisite for the development of IDDM. Infection of the surrounding tissue might lead to release of immune mediators like interferon-γ, TNF-α, and nitric oxide, which have been shown to be harmful to β cells in *in vitro* cultures ("bystander" death).[15,16]

The negative selection of T cells during their maturation in the thymus eliminates most autoreactive T cells that have a high affinity for self-determinants. Some autoreactive T cells evade negative selection because their affinity to the self-determinant in the thymus is too low. Bystander activation describes a process whereby autoreactive T cells are activated during an immune response against the viral infection by various mechanisms. The epitopes recognized by self-reactive and viral reactive T cells are different.

The proinflammatory cytokine milieu (TNF-α, interferon-γ, interleukin-12) generated during the antiviral immune response might improve the antigen uptake, antigen processing, and subsequent presentation of self-antigens by antigen presenting cells (APC) like dendritic cells, B cells, and macrophages. Together with cytokine-mediated overexpression of costimulator molecules on antigen presenting cells, this lowers the threshold for the activation of autoreactive T cells. This is important, as autoreactive T cells can usually be found in all humans, but they possess a high activation threshold due to the negative selection in the thymus during maturation.[17] There are also notions of MHC class I and even class II up-regulation on β cells during the autoimmune process, but several animal models have proved that a direct contact between β cell and autoreactive T cell is not necessary for the development of diabetes.[18]

Another mechanism of bystander activation of autoreactive T cells is the release of sequestered self-antigens during virus-induced destruction of β cells. Antigens that have been ignored by autoreactive T cells might gain access to the antigen-presentation pathways.[19–21]

According to the model of "molecular mimicry" an initial immune response against viral proteins sharing similarity with self-proteins would trigger autoreactive T and B cells, which can then destroy self-tissue even after clearance of the viral infection.

Although this model has been extensively used to explain the pathogenicity of most viruses implicated in IDDM (TABLE 1) by showing cross-reactive autoantibodies or cross-reactive T cells, formal proof of its relevance in human IDDM is still lacking, as will be discussed later.

Virally encoded superantigens (SAGs) might be another mechanism causing β cell destruction.[22] SAGs can cause an unspecific stimulation of a large T cell subset, possibly including autoreactive T cells. This might enable these cells to obtain access to peripheral tissues and to initiate autoimmunity. In this respect it is of interest to note that pancreatic tissue and spleen from newly diagnosed type I diabetics showed a preferential usage of the T cell receptor Vβ7-chain as a hint for possible SAG action.[23,24]

Although such a superantigen has been found to be encoded by a retroviral sequence isolated from diabetes patients,[23,24] it turned later out that this viral sequence had no disease association.[25,26]

We are just starting to understand the mechanisms counteracting T cell activation by regulatory T cells, inhibitory cytokines and other pathways.[27] It is well conceiv-

able that viral infection might alter this delicate balance between activation and inhibition, although formal proof for human IDDM is still missing.

Finally, it is becoming increasingly clear that the strength and specificity of an immune response are in part influenced by preceding immune responses. This can be explained by a different composition of the pool of memory cells[28–30] or by deletion of T cells involved in clearance of preceding viral infections or other environmental agents.

It has to be emphasized that besides the various possibilities of viral infections in triggering IDDM, autoimmunity is still a rare outcome of an antiviral immune response, possibly due to multiple regulatory pathways. But in a scenario in which multiple genetic polymorphisms lead to a dysregulation of the immune reaction, the above-mentioned pathomechanisms of viral infection may contribute to a further dysregulation and may thereby represent the final trigger of β cell destruction.

ENTEROVIRUS/COXSACKIE B VIRUS

Although various viruses of the enterovirus family have been implicated in IDDM, most studies found a particular association with Coxsackie virus infection.[31,32] Coxsackie viruses are enteroviruses belonging to the family of picornaviruses (small-RNA viruses). From the 23 Coxsackie A serotypes and the 6 B serotypes, the serotype B4 has been especially suggested to be involved in the etiology of human IDDM.

Beside several case reports of recent-onset IDDM with a preceding or concurrent Coxsackie B infection,[33] epidemiological studies[34] have shown a high frequency of IgM antibodies against Coxsackie B in children newly diagnosed with IDDM.[32,35] There are, however, also contradictory results, since some reports have been unable to find any differences in antibody prevalence and titer against Coxsackie virus[36] or even decreased levels of antibodies.[37] It is possible that detectable levels of autoantibodies to relative common enteroviruses in some individuals may actually represent increased humoral immunity linked to specific HLA genotypes or other IDDM susceptibility genes, and previous studies have not adequately adjusted case-control comparisons. None of the studies has taken into account the possible effect of polio vaccinations on the development of heterotypic antienterovirus antibodies, which is important given the cross-reactivity observed in all assays between different serotypes.[31] Furthermore, presence of antiviral antibodies does not prove causality given the long lag period between appearance of autoantibodies and clinical IDDM.

In more recent studies reverse transcriptase polymerase chain reaction (RT-PCR) has been applied to detect Coxsackie virus mRNA in sera of patients with newly diagnosed IDDM. In these retrospective studies children and adults with recent-onset IDDM were tested positive more frequently than control subjects.[38,39]

The tropism phenomenon (the characteristic of a virus to infect a particular tissue or cell type) is thought to explain why some viruses of the enterovirus family are diabetogenic and some are not.[40] Coxsackie B4 and B5 viruses have been isolated from the pancreas of patients with acute-onset IDDM, and the isolates were able to induce diabetes in susceptible mouse strains.[41] The possibility of the virus to home to islets has further been suggested by studies isolating viral antigens from islets of children with fatal viral infection.[42]

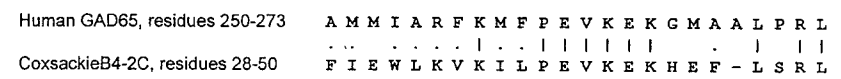

FIGURE 1. Sequence homology between human GAD65 and the 2C protein B4. *Lines* show identical amino acid residues, while *dots* indicate amino acid residues with similar charge, polarity, or hydrophobicity.

As nonhuman primates generally experience nonnapparent infections, most animal data rely on rodent models. Coxsackie B4 viruses and other serotypes can induce IDDM in certain mouse strains, if the virus has been passaged in cultured β cells in order to shift the tropism of the virus from pancreatic acinar to β cells.[43,44]

The exact mechanism of the diabetogenic action of Coxsackie virus infection is not clear, but experimental data supports different possibilities that might act synergistically in individual patients. As Coxsackie viruses possess strong cytolytic activity, they may contribute to direct β cell destruction, as has been shown in rodent models.[43,44] This may be especially relevant in the small subgroup of patients with a fulminant start of diabetes. Furthermore, infection with Coxsackie B4 virus increases the pancreatic expression of glutamic acid decarboxylase 65 (GAD),[45] a major autoantigen of the autoimmune response in humans and nonobese diabetic mice.[46–48] Although unproved, this mechanism might hypothetically lead to an increased uptake of GAD by antigen presenting cells and subsequent presentation to T and B cells, thereby reaching the threshold for activation of these cells.[17]

Molecular mimicry has also been postulated as a possible pathomechanism of Coxsackie virus infection in IDDM due to a sequence homology between the viral noncapsid protein P2-C and GAD.[49] Antibodies to P2-C (residues 35–46) were detected in serum of 10 IDDM patients, and 8 of these sera also reacted with GAD65 (residues 256–268).[50] However, it has later been shown by using monoclonal antibodies to GAD65 from a diabetic patient that although the region of GAD that is homologous to P2-C was the target of diabetes-associated autoantibodies, the antibodies showed no cross-reactivity with native viral antigens of Coxsackie virus B1-6, rubella, or cytomegalovirus.[51] Furthermore, IgM-positive human sera against Coxsackie B4 did not cross-react with recombinant GAD65.[17]

In addition to cross-reactive humoral epitopes, T cell responses to Coxsackie virus peptide P2-C aa 32–47 were detected in three individuals, who also showed a proliferative T cell response to GAD65 aa 260–279.[52] However, one has be very cautious not to misinterpret data on molecular mimicry.[53] It has still to be shown that possible cross-reactive epitopes are, indeed, generated and presented on antigen presenting cells during the immune response in diabetes or against the virus and that T cells against the respective GAD epitope are involved in β cell destruction. Furthermore, to rule out other possible pathomechanisms, the modification of the viral epitope should abrogate diabetes development in an experimental setting in animals. Therefore, molecular mimicry in diabetes is suggested, but by far not proved.

On the other hand molecular mimicry may be responsible for the conflicting serological results regarding antibody positivity to Coxsackie virus, which might rather represent cross-reactivities to GAD65.

Finally, bystander activation of autoreactive T cells during an immune response against Coxsackie virus might be possible (see TABLE 2). In addition to cytokine-

mediated activation of antigen presenting cells and/or autoreactive T cells, the local viral infection might cause an increased release of β cell antigens, which are subsequently presented in the "danger" context of a viral infection.

Indeed, bystander activation during infection with Coxsackie virus has been shown to be important in a mouse model in which diabetes was caused by a homogeneous population of β cell–reactive T cells (T cell receptor transgene) that did not cross-react with Coxsackie virus. In this model Coxsackie virus infection caused IDDM.[54] However, the high precursor frequency of autoreactive T cells in that particular transgene model makes comparisons to the human situation difficult, as autoimmune diseases are rather characterized by a small, heterogeneous autoreactive T cell population.[17,55]

Taken together, the few prospective studies performed to date suggest that Coxsackie viruses may trigger β cell autoimmunity or potentiate existing β cell autoimmunity.

RETROVIRUS

Retroviruses (RVs) are RNA viruses that contain reverse transcriptase. It is important to distinguish exogenous retroviruses, like most oncoviruses, lentiviruses, and spumaviruses, from endogenous retroviruses. While exogenous retroviruses are able to produce infectious particles and spread from organism to organism, endogenous retroviruses are part of the genome, and their genetic information is transmitted vertically via germline. Although there are endogenous RVs in rodents that are able to produce infectious viral particles, no such particles have been detected so far from retroviral sequences in the humane genome. In fact, about 5 to 10% of the mammalian genome consists of elements introduced by reverse transcription, and endogenous retroviral sequences encompass about 1% of the human genome.[56] Exogenous and endogenous retroviruses have been considered to play a role in inciting autoimmunity, and a great deal of misleading data about their influence in IDDM has been published in recent years. With more sensitive PCR-based methods an increasing number of genome fragments and reverse transcriptase activities of potential retroviruses were reported in humans and animals with autoimmune disorders.[57,58] Autoimmunity caused by retroviruses has been explained by molecular mimicry, immunomodulation and -maturation, or transactivation and insertional events. Finally, retrovirus-like particles were found in patients suffering from multiple sclerosis[59] and Sjögrens syndrome,[60] but not in healthy controls. The hypothesis of a retroviral etiology of autoimmune disease was further supported when a superantigen (SAG) was found to be encoded in the U3 region of the 3´ long terminal repeat (LTR) of the mouse mammary tumor virus.[61] Subsequently, a full-length genome of an endogenous retrovirus, termed IDDMK$_{1,2}$,[22] was isolated from cultivated islet cells of two patients with new-onset IDDM.[23,24] Further experiments revealed that the viral genome contained a superantigen in the N-terminal part of the envelope protein. Nevertheless, subsequent studies have shown that there is no disease association of this retroviral sequence and its mRNA transcripts with IDDM.[25,26] Furthermore, no retroviral particles were found. Although a retroviral influence on initiation of IDDM cannot be ruled out, substantial proof is still lacking for diabetes and other human autoimmune diseases. Altogether we have to be very careful with the interpretation

of data on endogenous human retroviruses in autoimmune diseases, as very sensitive molecular methods might generate artifacts due to the high prevalence of defective endogenous RVs in our genome.

Some endogenous RVs are able to transcribe RNA and generate proteins, and this activity is modulated by cytokines. Therefore, the detection of viral RNA transcripts and of antibodies against viral proteins in patients with autoimmune disease does not necessarily mean that a virus has caused the autoimmunity; it could rather be the consequence of an altered cytokine environment due to the autoimmune inflammation.

RUBELLA VIRUS

Approximately 12–20% of individuals infected *in utero* with rubella will subsequently develop diabetes within the next 5–20 years.[62,64] An autoimmune etiology is suggested, as islet cell and anti-insulin autoantibodies have been found in 20% of nondiabetic individuals and in 50–80% of patients with diabetes after congenital rubella infection. As antibodies against various rubella proteins cross-reacted with a 52-kDa β cell protein and a rat insulinoma cell line, molecular mimicry might play a role,[65] although similar limitations as discussed for Coxsackie virus molecular mimicry make the interpretation difficult. As the rubella embryopathy syndrome is now extremely rare due to effective population-based vaccination programs, the fetal infection does not account for the large number of IDDM patients.

In addition to the congenital rubella syndrome, single cases of IDDM after rubella infection in adults have been reported.[66] Experimentally, the rubella virus was able to cause diabetes in both rabbits and hamsters.[62,67]

CYTOMEGALOVIRUS

Single-case reports of IDDM after cytomegalovirus (CMV) infection have been reported in children[68] and adults.[69–71] More indirect evidence came from studies showing that 35% of newly diagnosed IDDM patients showed serological evidence of recent CMV infection and association with islet cell autoantibodies.[70,72–74] On the other hand a prospective study from Sweden screening 16,474 newborns for congenital CMV infection was unable to detect any correlation of CMV infection with diabetes.[75] As viral infections are most likely just triggering β cell autoimmunity in genetically susceptible individuals and as this group is rather small in the general population, future prospective studies should be done within this group of subjects that is at risk to develop IDDM.

CMV infection can cause β cell damage, including degranulation. Signs of infection of β cells have been shown in the form of characteristic inclusion bodies in β cells of children who died of disseminated CMV infection.[42] Besides this, 20% of IDDM patients appear to have CMV DNA in their islets.[70] In line with studies mentioned above, molecular mimicry has been suggested by antibody cross-reactivity between CMV proteins and an unidentified 38-kDa protein from pancreatic islets.[76] However, the few cases of CMV-associated IDDM reported and the high prevalence

of individuals with immunity to CMV make CMV unlikely to be a major reason for development of IDDM.

EPSTEIN-BARR VIRUS

A potential role of Epstein-Barr virus (EBV) infection has been suggested in several autoimmune diseases,[77] and single cases have been reported with a temporal relation of EBV infection and development of IDDM.[78,79] Due to sequence homology between the BOLF1 protein of EBV and an 11-amino acid residue of the human HLA-DQW8 β chain, molecular mimicry was postulated as a pathomechanism. However, sera from IDDM patients did not bind to either β chain or BOLF1.[80] Another sequence homology has been described between an adjacent region of the HLA-DQW8 β chain and the BERF4 protein of EBV. In a small retrospective study, two patients who developed a humoral immune response against BERF4 during acute EBV infection subsequently rapidly developed IDDM, while five other acutely infected patients without humoral reactivity to BERF4 did not become diabetic.[81] Although EBV might have precipitated IDDM in a minority of patients with IDDM, it is probably not a trigger in the majority of patients.

OTHER VIRUSES AND HUMAN IDDM

There are further anecdotal reports of development of IDDM after viral infection with Coxsackie A and echovirus,[40,82] hepatitis A,[83] varicella zoster,[84] measles,[85] mumps,[86–88] rotavirus,[89] polio,[85] and influenza.[40]

OTHER VIRUSES IN ANIMALS

Several other viruses are able to cause diabetes only in animals. These include encephalomyocarditis virus (EMC), reovirus, and mengovirus in mice; Kilham's rat virus in rats; and foot and mouth disease and bovine viral diarrhea-mucosal disease viruses in cattle. While certain strains of the EMC virus cause diabetes by direct infection and destruction of β cells in genetically susceptible mouse strains, Kilham's rat virus causes an immunological autoimmune response to the islets.

It is interesting that a single point mutation in the VP1 protein of the EMC virus determines β cell tropism and diabetogenicity of EMC viral strains.[90] Therefore, the genetic variability at the level of the receptor (on both host and virus) may be an important component of non-HLA-encoded genetic susceptibility.[91]

PREVENTION OF IDDM BY VIRAL INFECTION/VACCINATION

In marked contrast to the diabetogenic potential of some of the above-mentioned viruses, some viral infections are able to delay or prevent the development of diabetes. This context is difficult to study in humans, but there are several examples in rodent models. For example, lymphocytic chorionmeningitits virus (LCMV)[92,93] and

mouse hepatitis virus (MHV)[94] can protect spontaneous diabetic BB rats and NOD mice from diabetes. Astonishingly, the EMC virus D that is strongly diabetogenic in other mouse strains prevents diabetes in the spontaneous diabetic NOD model.[95] Identifying the underlying mechanisms in the rodent models might be very helpful for future therapies in humans.

It has been speculated that induction of antiviral antibodies might lead to a humoral anti-idiotypic immune response against autoreactive T cells. Furthermore, the induction of regulatory/suppressor T cells may be possible.[27] Recent data has demonstrated that a preceding viral infection can shape our pool of memory T cells, which results in a different immune response to subsequent antigen exposure.[28] It is conceivable that such influences on our immune repertoire are also important for the development of immune responses against self-antigens.

As mentioned above for infection with enteroviruses in humans and EMC virus in rodents, there are probably only distinct strains of the same virus, which show β cell tropism in genetically predisposed individuals. Therefore, an early infection or vaccination with a nondiabetogenic strain of the virus could induce immunity against antigenically similar diabetogenic strains, thereby protecting from autoimmunity and IDDM. Before this approach can be used therapeutically, additional studies will be needed to examine interactions between viral infections and non-HLA IDDM candidate genes, including those that may determine β cell tropism of diabetogenic viral strains, if such exist.

In addition to a possible protective effect, vaccination against viruses might theoretically also trigger autoimmune responses. In fact, vaccination is the only way to achieve a disease resembling multiple sclerosis in rodents. Nevertheless, in humans there is no hint that the mumps-measles-rubella vaccination programs have resulted in IDDM.[96]

TRANSGENIC ANIMAL MODELS OF DIABETES

A more mechanistic understanding of the events potentially leading to virally mediated IDDM has evolved from studies using transgenic animal models.[19–21,97–99] Among the main players are indirect, bystander activation and molecular mimicry, explained above.

Two transgenic mouse models were developed in our laboratory, in which expression of a viral "self-antigen" (later used to prime an autoimmune, "antiviral" response by systemic infection with the autologous virus) was either directed to oligodendrocytes (myelin basic protein promoter, MBP) or pancreatic β cells (rat insulin promoter, RIP). Since the viral gene is incorporated as a "transgene" into the host's genome and is passed on to the progeny, it becomes a part of the immunologic "self," when it is expressed early enough during development. Thus the viral transgene becomes essentially a traceable self-antigen that can be targeted in an autoimmune process. Infection with the autologous virus (LCMV) induces immunopathology (infiltration by lymphocytes, activation of antigen presenting cells, and expression of inflammatory cytokines) in both strains of mice; but target cell destruction and autoimmune disease occur only in RIP-LCMV transgenic mice (autoimmune diabetes),[19] whereas significant demyelination does not develop in MBP-LCMV transgenic mice.[100] This obvious difference in disease severity be-

tween the two transgenic mouse models is by itself quite interesting. It is found despite the fact that equal numbers of autoreactive lymphocytes enter the target organ and accumulate there (CNS or pancreatic islets). Thus, the central nervous system has some strong, intrinsic protective mechanism—for example, the predominance of certain cytokines, such as TGF-β, that are generally immune down-regulatory. Alternatively and maybe more likely, the reason for this discrepancy is the fact that LCMV, when triggering the autoimmune process in both strains of transgenic mice, readily infects the pancreas but not the brain. Through the local viral presence APCs are directly activated and express viral "self" antigens that can then propagate the local activation and amplification of autoreactive (self-transgene–specific) lymphocytes. Without this extra "boost," arrival of autoreactive lymphocytes in the target organ is insufficient to cause disease (as it is the case in the CNS model), and these soon loose "steam," though remaining in the organ in a nonactivated stage. This has been demonstrated for the RIP-LCMV model as well, by transferring autoreactive lymphocytes (and not infecting with the virus), in which case no IDDM developed.[101] These findings imply for other autoimmune diseases that local damage by a virus might precipitate and enhance autoimmune processes.

In the following paragraphs we will describe three novel paradigms derived from experiments with our two transgenic models, which illustrate the complexity of virally induced autoimmune disease and explain why most, if not all, approaches to detecting a viral etiology for autoimmune diseases in humans have failed so far.

First, in both transgenic models, disease (autoimmune diabetes) or immunopathology (CNS inflammation) develops only long after the viral infection has been eliminated. Whereas the first entry into the pancreatic islets or the CNS of autoreactive lymphocytes specific for the transgene coincides with the peak of the systemic, primary antiviral (LCMV) lymphocyte response *in vivo*, local infiltration into the target organ builds up much later (2 weeks up to 4 months post-LCMV infection) and continues long after the infection has been cleared. This finding implies that local presence of a self-antigen (or a persisting viral protein) presented by APCs in the target organ, but not viral nucleic acids or live virus, is required to "drive" an autoimmune process to cause tissue injury and disease. However, more importantly, clear "traces" of the preceding viral infection are not necessarily found in the target organ. Thus, potentially the only remaining marker might be the antiviral immune response. Unfortunately, even using antibodies or lymphocyte proliferation assays in detecting a viral etiology for autoimmune diseases is problematic, as will become clear from the following two considerations.

When different strains of the autologous virus (LCMV) that normally causes disease and cross-reacts with the self-antigen at the T and B cell levels are used to initiate disease, some do but others do not cause autoimmune diabetes. The underlying reason is the variation in numbers of antiself (viral) T cells generated, since some of the cross-reactive viral strains have mutations in the T cell peptide epitope(s), while others have a single amino acid mutation outside the epitope in the flanking sequence, which impairs antigen presentation and in this way makes the generation of autoreactive effector T cells less efficient. Hence, since RNA viruses exist as "swarms" and within a given viral population/quasispecies variants will exist that cause disease or may not do so, conventional molecular probing and serologic or T cell proliferation assays may fail to discriminate between disease-producing and

non-disease–producing viruses and cannot be used to clearly identify one virus as the etiologic trigger of a given autoimmune disease.

Last, once the disease process has been initiated by the viral strain autologous to the "self"-protein (transgene), infection with a heterologous virus can enhance the autoimmune process, leading to much more rapid disease development (diabetes model) or increased immunopathology (CNS model). In these scenarios, no evidence for cross-reactive T cell epitopes exists. Thus, bystander activation of autoreactive lymphocytes involving inflammatory cytokines, chemokines, and other still-unknown molecular interactions (for example, implicating APCs) must be postulated, a paradigm we are currently investigating. As a consequence, the multitude of viral infections each individual encounters in his/her lifetime can overshadow and modulate a single causative process. It is, furthermore, important to add that we are so far unable to predict the degree of bystander activation a virus is able to induce, because the underlying molecular mechanism(s) are still elusive.

CONCLUSIONS

In conclusion, studies from monozygotic twins in multiple sclerosis as well as type 1 diabetes showing clear disease discordance strongly point toward an environmental/infectious etiology in addition to genetic predisposition. Nevertheless, many of the above-mentioned viruses have also been implicated in the initiation of other human autoimmune diseases, indicating that none is so far disease specific. It might rather be that the genetic background and the sum of environmental influences, which shape the immune repertoire, might predispose individual patients to be more susceptible to the diabetogenic potential of distinct viruses.

Proving an influence of a certain virus on the development of IDDM in humans is further hampered by the long interval between the start of autoimmune reactions and clinically apparent diabetes. Due to the fact that viral infections will likely be cleared at later stages of disease progression, testing for viral presence or specificity of autoreactive lymphocytes within the target organ (CNS, pancreas, etc.) will have to be done early, around the time of disease induction. This is difficult to achieve, and therefore viruses have been (and will be) isolated from affected organs only if a persistent infection is the cause (i.e., certain herpes viruses for MS) or the induction of disease is hyperacute (i.e., Coxsackie B4 virus in certain cases of early-onset childhood diabetes). Further, serological screenings are mostly inadequate to establishing a correlation, because strains of the same virus with identical antibody profiles could or could not induce disease. We would propose that, based on our findings and these considerations, a potential viral etiology has to be proved by obtaining tissue samples from individuals at risk, testing for viral antigens, and assessing specificities of autoreactive T cells in the target organ. This should be part of a large prospective study in a cohort of genetically predisposed individuals and should involve a large panel of known pathogens. Based on our present knowledge, this should either rule out or prove a viral etiology for autoimmune diseases.

Finally, viral infections may be involved in the pathogenesis of IDDM by acting as the terminal insult in individuals who have already lost a substantial β cell mass through ongoing autoimmune damage. Further destruction of remaining β cells might then result in the earlier clinical onset of IDDM.

To date no single infectious agent has been shown to be responsible for the development of IDDM. Therefore, vaccination studies are at the moment not likely to be successful in preventing development of diabetes.

Regardless of the cause of diabetes, MS, or other autoimmune syndromes, antigen-specific treatments using self-reactive lymphocytes are a possibility. For these strategies, the initiating autoantigens do not have to be known, but self-antigens implicated later in the autoimmune process have to be identified. Regulatory lymphocytes recognizing these antigens can be induced by their oral administration or DNA vaccines and have the ability to home on or be retained in the affected organ, because they locally recognize their respective autoantigen. There, they secrete a regulatory factor or cytokine—for example, IL-4—that locally reduces the activation and expansion of autoreactive destructive cells of various specificities and thus prevents disease development.[13] Such approaches are currently under investigation in our laboratory and have the promising potential to become a treatment for several autoimmune disorders, once the specific antigenic and immunization requirements, as well as the mechanism(s) modifying regulation, have been precisely defined. In contrast to this strategy aimed at the induction of autoreactive regulatory lymphocytes, the converse approach, which is the selective systemic reduction or elimination of autoreactive inflammatory cells, has to surmount more obstacles and is more problematic. First, the majority of the main inflammatory autoreactive T cell epitopes involved early in disease have to be mapped, which is difficult to achieve in an outbred population. Second, epitope and antigenic spreading can occur later in disease, which may interfere with the success of this strategy. However, it is possible that antigen-specific interventions directed to only one self-epitope, when applied early enough, can abrogate an entire autoimmune process.[102]

Future prospective, multicenter studies in patients with an increased risk of developing IDDM and research in animal models will, it is hoped, improve our understanding of the interaction of environmental factors in genetically predisposed individuals.

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